AWARD NUMBER: W81XWH-13-1-0184

TITLE:
In Vivo Tagging of Lung Epithelial Cells To Define the Early Steps of Tumor Cell Dissemination

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CONTRACTING ORGANIZATION:

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REPORT DATE: October 2014

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2014	Annual	15Sep2013-14Sep2014

4. TITLE AND SUBTITLE

In Vivo Tagging of Lung Epithelial Cells To Define the Early Steps of Tumor Cell Dissemination

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0184

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Trustees of Boston University, BUMC
Office of Sponsored Programs
85 East Newton Street, M-921
Boston, MA 02118

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

To understand the early events that accompany invasive behavior in vivo, we proposed to develop a lineage-labeling system to detect and isolate cells of lung epithelial origin during tumor progression in a mouse model of lung cancer. A mouse model of lung cancer with metastasis [LSL-$Kras^{G12D}$/$Lkb1^{L/L}$ mice] was interbred with Nkx2.1-$CreERT_2$ knock-in mouse strain, containing a tamoxifen-inducible lineage specific Cre recombinase, to generate adult lung-specific mutations in Kras and Lkb1. We introduced a $Rosa^{YFP}$ (lox-stop-lox-YFP) into the mutant background to specifically label and track lung epithelial cells during tumor progression and metastasis. This model ($Kras^{G12D}$/$Lkb1^{L/L}$/$YFP^{L/-}$/Nkx2-1creER2$^{+/-}$ mice), will allow us to determine the timing of dissemination during the natural evolution of lung adenocarcinoma in vivo and correlate cell phenotype with the acquisition of invasive and tumor-initiating properties. We will test our hypothesize in year 2 (NCE) that (1) lung tumor cells invade and disseminate early in tumor evolution via EMT and (2) disseminated cancer cells exhibit a stem cell-like phenotype. Successful completion of this project promises to shift the current paradigm of lung cancer metastasis and will aid in early detection, and novel treatment approaches.

15. SUBJECT TERMS

Epithelial mesenchymal transition, lung cancer, metastases, mouse models of lung cancer, lineage-labeling

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. INTRODUCTION: Despite the high prevalence and poor prognosis of lung cancer, little is known about the mechanisms of progression. There are two major metastasis paradigms: In the classical model, tumors acquire mutations that promote invasive behavior and dissemination late in tumor evolution, whereas in the alternative model, metastasis is an inherent feature of tumors very early in its natural history. A challenge in studying tumor cell dissemination has been the identification of markers that can distinguish cancer cells from cells that normally reside in the bloodstream or at sites of seeding. As a result, there remains uncertainty regarding the mechanisms of metastasis as well as the timing of dissemination. Epithelial-mesenchymal transition (EMT), a process by which cells lose epithelial characteristics and develop mesenchymal properties, has been implicated as a means by which tumor epithelial cells acquire the ability to invade and disseminate. Most studies of EMT in the context of cancer biology, however, have been based on cultured cells manipulated in vitro, and thus the relevance of EMT to in vivo carcinogenesis is controversial. If EMT is an early process, then detection methods that rely on cellular expression of epithelial markers alone are unlikely to provide a complete picture of metastasis. To understand the early events that accompany invasive behavior in vivo, we proposed to develop a novel, precise, and sensitive lineage-labeling system to detect and isolate cells of lung epithelial origin during tumor progression in a mouse model of lung cancer. This system will allow us to determine the timing of dissemination during the natural evolution of lung adenocarcinoma in vivo and correlate cell phenotype with the acquisition of invasive and tumor-initiating properties.

2. **KEYWORDS:** Epithelial mesenchymal transition, lung cancer, metastases, mouse models of lung cancer, lineage-labeling, Kras, Lkb1, Nkx2-1creER2$^{+/-}$ mice.

3. **OVERALL PROJECT SUMMARY:**

We hypothesized that (1) lung tumor cells invade and disseminate early in tumor evolution via EMT and (2) disseminated cancer cells exhibit features of tumor propagating cells. We proposed to test our hypothesis by developing a sensitive method to lineage-tag and track lung epithelial cells in a mouse model of metastatic lung cancer.

To test our hypothesis, we proposed to develop a mouse model of lung cancer with metastasis driven by conditionally activated Kras and concurrent tumor suppressor Lkb1 loss [LSL-Kras^{G12D}/Lkb1$^{L/L}$ mice] (3). Nkx2.1-CreERT$_2$ knock-in mouse strain (4), containing a tamoxifen-inducible lineage specific Cre recombinase, was interbred with LSL-Kras^{G12D}/Lkb1$^{L/L}$ mice to generate adult lung-specific mutations in Kras and Lkb1, genes that are mutated with high frequency in human lung adenocarcinomas. We introduced a Rosa^{YFP} (lox-stop-lox-YFP) into the mutant background to specifically label and track lung epithelial cells during tumor progression and metastasis. Because the Nkx2.1 (TTF1) promoter is active only in endoderm-derived lung cells, only the epithelium will be tagged by this method. Prior to using these animals for our studies, we will ensure that lineage labeled mice display similar histology as non-labeled mice and that mesenchymal cells are not labeled under control conditions in Nkx2.1-CreERT$_2$ animals.

In our Statement of Work Task 1 as stated below has been completed. It took approximately 12 months to complete task 1.

Task 1.

Generate Kras ^{G12D}/Lkb1$^{L/L}$/YFP$^{L/-}$/Nkx2-1creER2$^{+/-}$ mice (timeframe months1-10 by Drs Kathuria, Cao, Ramirez).

1. We will follow a specific breeding plan to obtain Kras ^{G12D}/Lkb1$^{L/+}$/YFP$^{L/-}$/Nkx2-1creER2$^{+/-}$ mice. We currently have in our facility Kras ^{G12D}/Lkb1$^{L/L}$, YFP$^{L/L}$, and Nkx2-1creER2$^{+/-}$ lines (timeframe: months 1-6 by Kathuria/Cao).
2. Kras ^{G12D}/Lkb1$^{L/+}$/YFP$^{L/-}$/Nkx2-1creER2$^{+/-}$ mice will be finally bred to generate Kras ^{G12D} /Lkb1$^{L/L}$/YFP$^{L/-}$/Nkx2-1creER2$^{+/-}$ mice (timeframe: months 6-10 by Kathuria/Ramirez).
3. Crossings will be set up at the LASC animal facility and tails will be genotyped by PCR (timeframe: months 1-10).

Mouse model development. We have successfully completed task 1 and have developed a novel, precise, and sensitive lineage-labeling system to detect and isolate cells of lung epithelial origin during tumor progression in metastatic and non-metastatic mouse models of lung cancer.

Kras is mutated with high frequency in human lung adenocarcinomas. Lkb1 is a tumor suppressor gene that is mutated in 30% of lung cancers (Fig 4). We used a mouse model of lung cancer with metastasis driven by conditionally activated Kras and concurrent tumor suppressor Lkb1 loss [$Kras^{G12D\ L/-}$/$Lkb1^{L/L}$ mice]. $Kras^{G12D\ L/-}$/$Lkb1^{L/L}$ mice develop tumors within 4 weeks of intratracheal adeno-Cre activation and die within 8 to 10 weeks, with local and distant metastases. Although $Kras^{G12D\ L/-}$/$Lkb1^{L/L}$ mice develop both adenocarcinomas and squamous cell carcinomas, all metastatic foci appear to be adenocarcinoma in origin.

A similarly derived model that does not metastasize activated by $Kras^{G12D}$ alone [$Kras^{G12D\ L/-}$] was also used for our studies. $Kras^{G12D\ L/-}$mice, which develop only primary lung tumors, develop a mixture of adenomas and adenocarcinomas 16 weeks after intratracheal adeno-Cre activation of oncogenic Kras and die within 24 to 26 weeks.

We are currently breeding these mouse models (Fig 1,2). First, we intercrossed the model of lung cancer with metastasis driven by conditionally activated Kras and concurrent tumor suppressor Lkb1 loss [$Kras^{G12D\ L/-}$/$Lkb1^{L/L}$ mice] to the Nkx2-1-CreER knock-in mouse strain, containing a tamoxifen-inducible lineage specific Cre recombinase, in a Rosa-YFP (lox-stop-lox-YFP) background [Nkx2-1- $CreER^{+/-}$ ER/Rosa $YFP^{L/L}$ mice]. This model has generated adult lung-specific mutations in Kras and Lkb1, mutations commonly found in human lung adenocarcinomas, while activating lineage tracing. Second, we have generated a model of lung cancer without metastasis driven by conditional activated Kras only [$Kras^{G12D\ L/-}$], in the above lung epithelial, tamoxifen-inducible lineage tracing background [Nkx2-1- $CreER^{+/-}$ ER/Rosa $YFP^{L/L}$]. These models will allow specific labeling and tracking of lung epithelial cells during tumor progression and metastasis.

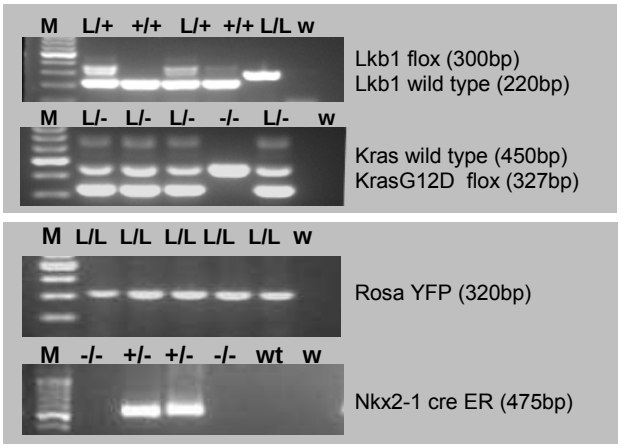
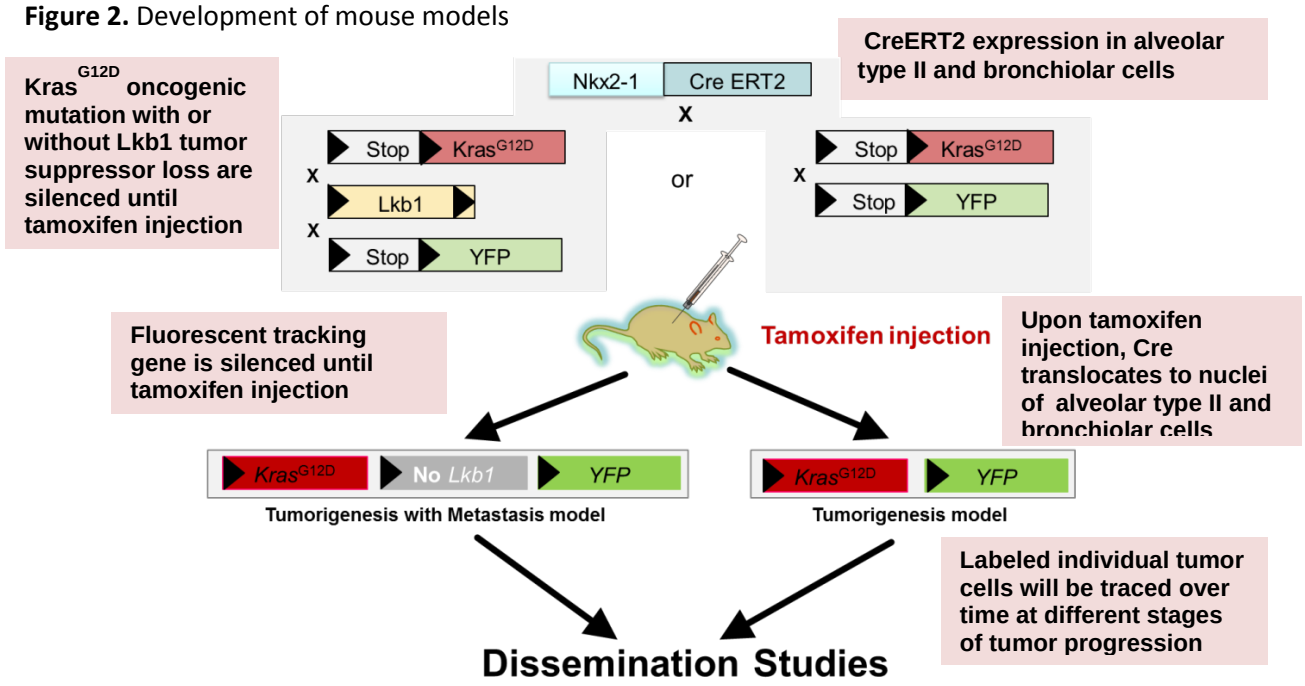


Figure 1. Genotyping of the $Kras^{G12D}$, Lkb1, Rosa YFP and Nkx2-1 Cre ER mice by PCR analysis.

Figure 2. Development of mouse models



Because the Nkx2-1 (TTF1) promoter is active in bronchiolar and alveolar type II cells, these tagged cells will be the precursors of the tumors by this method. Prior to using these animals in our studies, we will ensure that lineage labeled mice display similar histology as non-labeled mice and that mesenchymal cells are not labeled under control conditions [Nkx2-1- CreER$^{+/-}$ ER/Rosa YFP $^{L/L}$ animals]. We will also determine the influence of Nkx2-1 haplo-insufficiency on our two models. It has previously been shown that haplo-insufficiency of Nkx2-1 in a mutant Kras background causes non-metastatic mucinous adenocarcinomas. We therefore expect that since the Kras$^{G12D\ L/-}$/Lkb1$^{L/L}$model is in a Nkx2-1 haplo-insufficient background, we may observe fewer squamous carcinomas and more mucinous adenocarcinomas. Since all metastatic foci in the Kras$^{G12D\ L/-}$/Lkb1$^{L/L}$model appeared to be adenocarcinomas, we may see a more aggressive pattern of metastasis in our model.

We have applied for and have been granted a no cost extension until 9/14/2015. We are now working on Task 2 and 3 which will be completed in year 2.

Task 2. Isolate lungs for histology and immunofluorescence analyses (SA1a-b) and blood for flow cytometry analysis (SA2a-b) (timeframe months 6-10 by Drs Kathuria, Cao, Ramirez, and Kotton).

1. First we will induce tumors by tamoxifen injection of Kras ^{G12D}/Lkb1$^{L/L}$/YFP$^{L/-}$/Nkx2-1creER2$^{+/-}$ mice and creER2 negative controls (timeframe: months 6-10 by Dr. Ramirez/Cao).
2. We will collect lung tissues at three time points (2, 4 and 8 weeks) for pathology, H&E and immunofluorescence analyses (timeframe: months 6-9 by Dr. Kathuria/Ramirez).
3. Blood will be collected by heart puncture for flow cytometry analysis of YFP^{+} circulating tumor cells (timeframe: months 6-10 by Drs. Kathuria/Kotton).

Task 3. In a second round of breading we will isolate lungs and blood (at the same time points described above) after tamoxifen injection for flow cytometry analyses of single-cell suspensions for qRT-PCR analysis and in vitro assays (SA1c, 2b)(timeframe: months 9-12 by Drs Kathuria, Cao, Ramirez, Kotton.).

1. YFP^{+} E-cadherin $^{+}$, YFP^{+} E-cadherin- and YFP^{-} cells will be collected for RNA purification and qRT-PCR analyses and for in vitro analysis of self-renewal, clonality and survival properties (timeframe months 9-12 by Drs. Ramirez/Cao).
2. Blood will also be collected as above for flow cytometry analysis of YFP^{+} circulating tumor cells (timeframe months 9-12 by Drs. Kathuria/Kotton)

Tasks 2 and 3 will support SA1 and SA2 which are as follows:

Specific Aims

SA1. Using epithelial lineage tracing in a mouse model of lung cancer metastasis, characterize the temporal and spatial pattern of tumor cell dissemination and determine whether this process is associated with EMT.

a. Characterize the time course of development of intraepithelial neoplasias, primary tumors, and metastasis by H&E analysis and determine whether tumor cells co-express the YFP lineage-tag together with epithelial (E-cadherin, Nkx2.1) or mesenchymal (Zeb1, FSP1, N-cadherin, Twist1) markers by immunohistochemistry.
b. Using YFP epithelial lineage labeling, determine if cancer cells undergo EMT by identifying whether YFP^{+} cells (1) transverse the basement membrane, (2) co-express mesenchymal markers and/or lack E-cadherin expression, and (3) change to fibroblast-like morphology.
c. By qRT-PCR analysis of sorted YFP^{+} cells at various time points in tumor progression, determine at the transcriptional level if and when epithelial derived tumor cells activate a mesenchymal program.

In year 2, we will characterize the time course of development of precancerous intraepithelial neoplasias, primary tumors, invasion, and metastasis by H&E staining and microscopic analysis and determine by immunofluorescence and confocal microscopy whether tumor cells co-express the YFP lineage-tag with epithelial (E-cadherin, Nkx2-1) or mesenchymal (Zeb1, FSP1, N-cadherin, Twist1) markers (Fig 3). It has been reported that Kras$^{G12D\ L/-}$/Lkb1$^{L/L}$ mice develop tumors within 4 weeks of adeno-Cre activation and die within 8

to 10 weeks, with local and distant metastases (12). Kras ^{G12D}/Lkb1$^{L/L}$ mice that are YFP epithelial lineage labeled will therefore be sacrificed at 2, 4, 6, and 8 weeks after activation. Kras ^{G12D} mice will be analyzed at similar time points. We will compare Kras $^{G12D\ L/-}$ Lkb1$^{L/L}$; RosaYFP$^{L/-}$;Nkx2-1-CreER$^{+/-}$ lungs to two different controls: (1) Kras $^{G12D\ (-/-)}$Lkb1$^{+/+}$; RosaYFP$^{L/-}$;Nkx2-1-CreER$^{+/-}$ and (2) Kras $^{G12D\ L/-}$Lkb1$^{L/L}$; Rosa-YFP$^{L/-}$;Nkx2-1-CreER$^{-/-}$.

Upon tamoxifen injection Kras$^{G12D\ L/-}$Lkb1$^{L/L}$; Rosa-YFP$^{L/-}$;Nkx2-1- CreER$^{+/-}$mice will activate mutant Kras, harbor complete loss of Lkb1 and activate YFP in lung epithelial bronchiolar and alveolar type II cells, starting the tumorigenesis process. In the first control, mice will have wild type Kras and Lkb1 while activating YFP in lung epithelial bronchiolar and alveolar type II cells. The second control will receive tamoxifen but as they are Nkx2-1-Cre ER $^{-/-}$ no recombination will occur in any of the other floxed alleles. The same strategy will be applied to the Kras^{G12D}/ Rosa-YFP/Nkx2-1-CreER model.

Using YFP epithelial lineage labeling, we will determine if cancer cells undergo EMT *in vivo* by identifying whether YFP^{+} cells (1) transverse the basement membrane, (2) co-express mesenchymal markers and/or lack E-cadherin expression (Fig 3), and (3) change to fibroblast-like morphology. EMT is characterized by loss of epithelial markers (E-cad) and expression of mesenchymal markers (Zeb1, FSP1, N-cad, and Twist1).

SA2. Determine if circulating lung-derived cells have features of tumor propagating cells.

a. Characterize the time course of when YFP^{+} tumor cells enter the bloodstream by flow cytometry and determine whether YFP^{+} circulating cells exhibit an epithelial, mesenchymal, or mixed phenotype.
b. Determine whether circulating lung cells exhibit features of tumor propagating cells by determining if YFP^{+} circulating lung cells have self-renewal properties, clonal growth and survival.

The majority of patients with lung cancer have advanced stage disease at the time of diagnosis. Recent studies in prostate, breast and esophageal cancers have indicated that entry of tumor cells into the bloodstream may not be a late event in tumor progression as previously believed (7,19-21). In SA2, we will determine when tumor cells first enter the bloodstream, if circulating epithelial cells have features of tumor propagating cells, and if cancer cells undergo MET upon arrival to distant sites to allow metastatic growth.

We will characterize the time course of when YFP^{+} circulating cells are first detected in blood by flow cytometry analysis and determine whether these YFP^{+} cells express transcripts for epithelial and mesenchymal markers by qRT-PCR (Fig 4). We will isolate YFP^{+} cells from blood and culture in attachment-free conditions to determine *in vitro* if circulating cancer cells are capable of self-renewal properties, clonal growth and survival.

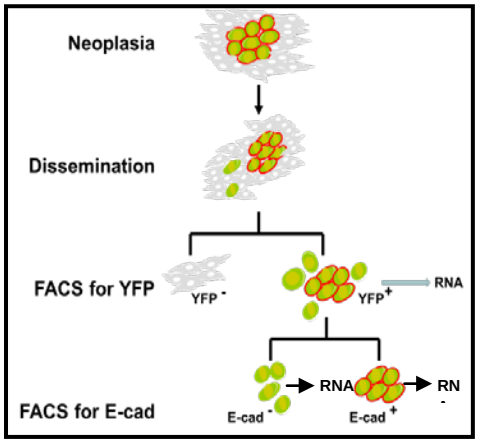


Figure 4. Strategy for isolating different tumor cell populations from the model mouse lungs.

4. KEY RESEARCH ACCOMPLISHMENTS:

We have generated generated Kras ^{G12D}/Lkb1$^{L/L}$/YFP$^{L/-}$/Nkx2-1creER2$^{+/-}$ mice which will be invaluable in completing the proposed aims to determine the timing of metastases and whether EMT occurs *in vivo*.

5. CONCLUSION:

To understand and reveal the early events that accompany invasive behavior *in vivo*, we have successfully completed Task 1 and have developed a novel, precise, and sensitive lineage-labeling system to detect and isolate cells of lung epithelial origin during tumor progression in metastatic and non-metastatic mouse models of lung cancer. These systems will allow us in year 2, to determine the timing of dissemination during the

natural evolution of lung adenocarcinoma *in vivo* and correlate cell phenotype with the acquisition of invasive and tumor-initiating properties. While other lung epithelial lineage tagging models have been used to determine the tumor cell of origin [$Kras^{G12D\ L/-}$/ /Sftpc-creER or $Kras^{G12D\ L/-}$/ /$p53^{L/L}$ /Sftpc-creER] (10), or the contribution of alveolar type II cells in injury-repair and regeneration models [Sftpc-creER or Scgb1a1-creER and Rosa-reporter lines] (11), no previous model has tracked lung epithelial cells during dissemination and metastasis. Successful completion of this project promises to shift the current paradigm of lung cancer metastasis and will aid in early detection and novel treatment approaches.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS: Nothing to report, but we anticipate several abstracts, publications, and presentations in the next year now that our model has been developed.

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report

8. REPORTABLE OUTCOMES: We will report and publish the generation of our mouse model once we have fully characterized it.

9. OTHER ACHIEVEMENTS: We will apply for further funding once we have performed the proposed experiments in year 2.

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11. **APPENDICES:** none